The ATPase activity of an ‘essential’ *Bacillus subtilis* enzyme, YdiB, is required for its cellular function and is modulated by oligomerization

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Characterization of ‘unknown’ proteins is one of the challenges of the post-genomic era. Here, we report a study of *Bacillus subtilis* YdiB, which belongs to an uncharted class of bacterial P-loop ATPases. Precise deletion of the *ydiB* gene yielded a mutant with much reduced growth rate compared to the wild-type strain. In vitro, purified YdiB was in equilibrium among different forms, monomers, dimers and oligomers, and this equilibrium was strongly affected by salts; high concentrations of NaCl favoured the monomeric over the oligomeric form of the enzyme. Interestingly, the ATPase activity of the monomer was about three times higher than that of the oligomer, and the monomer showed a \( K_m \) of about 60 \( \mu \)M for ATP and a \( V_{\text{max}} \) of about 10 nmol min\(^{-1}\) (mg protein\(^{-1}\)) (\( k_{\text{cat}} \sim 10 \text{ h}^{-1} \)). This low ATPase activity was shown to be specific to YdiB because mutation of an invariant lysine residue in the P-loop motif (K41A) strongly attenuated this rate. This mutant was unable to restore a normal growth phenotype when introduced into a conditional knockout strain for *ydiB*, showing that the ATPase activity of YdiB is required for the *in vivo* function of the protein. Oligomerization was also observed with the purified YjeE from *Escherichia coli*, a YdiB orthologue, suggesting that this property is shared by all members of this family of ATPases. Importantly, dimers of YdiB were also observed in a *B. subtilis* extract, or when stabilized by formaldehyde cross-linking for YjeE from *E. coli*, suggesting that oligomerization might regulate the function of this new class of proteins *in vivo*.

**INTRODUCTION**

With the advent of the post-genomic era, the scientific community has to face the tremendous challenge of assigning a biological role for each protein in every organism (Galperin & Koonin, 2004). This task is even much more demanding for the uncharted (or unknown) proteins because they are a priori unrelated to any previously characterized cellular function (White, 2006), and it has been estimated that these unknown proteins account for approximately one-third of each genome (Roberts, 2004; Tatusov et al., 2000).

In most living cells, one of the major classes of proteins is the P-loop (or Walker A motif: G/AXGKT/S; Walker et al., 1982) NTP hydrolase, since it amounts to 10–18 % of all gene products (Koonin et al., 2000). These proteins are quite versatile, interact with many different classes of proteins of different folds, and often play essential roles in the cell (Brown, 2005; Park et al., 2001; Yoshida & Amano, 1995). As such, uncovering the role and functional mechanism of newly uncharted bacterial proteins with a P-loop motif, not found in humans and widely distributed in the prokaryotic kingdom, is an important first step in the search for new antibacterial compounds (Brown & Wright, 2005). One family of proteins that suits these criteria and for which little information is yet available is the Uncharacterized Protein Family (UPF) 0079 of the Swiss-Prot database (http://www.expasy.org/). This family is represented by YdiB in *Bacillus subtilis* or YjeE in either *Escherichia coli* or *Haemophilus influenzae*. Recently YjeE from *H. influenzae* has been targeted in a search for new antimicrobial compounds (Lerner et al., 2007). Early investigations suggested that this protein was essential in *E. coli* and in *B. subtilis* (Allali-Hassani et al., 2004; Freiberg et al., 2001; Kobayashi et al., 2003); however, more recently, the ‘essentiality’ of ydiB in *B. subtilis* has been questioned (Hunt et al., 2006). In *H. influenzae*, the intracellular concentration of YjeE was high enough to be
detected by a proteomic approach (Langen et al., 2000). YjeE purified from either E. coli or H. influenzae possessed a very low ATPase activity (Allali-Hassani et al., 2004; Teplyakov et al., 2002), and as part of a structural genomic project, the 3D structure of YjeE from H. influenzae was solved either in an empty form (apoenzyme) or in complex with ADP-Mg (Teplyakov et al., 2002). Apart from the classical P-loop motif found in many NTP hydrolases (Geourjon et al., 2001; Saraste et al., 1990), this protein displayed a unique ATP-binding fold unrelated to any known ATPases or GTPases and thus formed a new family of enzymes (Teplyakov et al., 2002). Recently, it has been shown that a high copy number of rstA, encoding an uncharacterized response regulator, was able to suppress the growth phenotype of YjeE-depleted cells in E. coli, although the functional connection between the two proteins is still unclear (Campbell et al., 2007).

Here, we have revisited the dispensability of ydiB in B. subtilis with the construction of both a conditional knockout and a deletion mutant. In addition, YdiB was overexpressed in E. coli and purified to homogeneity by a three-step procedure, and the properties of the protein were studied. In particular, YdiB was shown to elute from a size-exclusion chromatography column as a heterogeneous mixture of monomers, dimers and higher molecular mass oligomers and this propensity to oligomerize was confirmed by different techniques. Pure recombinant YdiB exhibited a low, but significant, ATPase activity that was abrogated by a mutation of an invariant lysine in the Walker A motif. Importantly, this mutant was unable to complement a conditional knockout mutant of ydiB, showing that the cellular role of YdiB is directly linked to its ATPase activity. Interestingly, the oligomerization of YdiB affected its ATPase activity, and in vivo experiments on either YdiB or its ortholog from E. coli, YjeE, which also formed oligomers in vitro, support the likelihood that this process might also occur in vivo.

METHODS

General methods. E. coli and B. subtilis strains were grown in LB (Luria–Bertani) medium. Ampicillin was used at a concentration of 100 μg ml⁻¹. Antibiotic concentrations (μg ml⁻¹) for B. subtilis were: chloramphenicol, 10; spectinomycin, 150; and kanamycin, 25. All antibiotics were obtained from Sigma-Aldrich. Polymerase enzymes were obtained from Stratagene (Pfu) or New England Biolabs (Vent). Gel extraction kits and plasmid midi-prep kits were purchased from QBiogene and restriction enzymes were obtained from Fermentas. Western chemiluminescence reagents were obtained from Pierce. Anti-YdiB antibodies were obtained from Cocalico Biologicals and donkey anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Amersham. To detect His-tagged proteins, the SuperSignal West Pico HisProbe kit (Pierce) was used. Immobilon-P membranes were from Millipore. All other compounds were from Sigma-Aldrich.

Construction of disrupted and conditional knockout mutants of ydiB, and growth curves. The conditional knockout mutant was constructed by insertion of a second copy of ydiB at the amyE locus using the plasmid pSWEET (Bhavsar et al., 2001b), and by replacing ydiB with a spectinomycin-resistance cassette. The complementing copy is under the control of a xylose-inducible promoter. The ydiB-disrupted mutant strain was obtained by transformation of the wild-type B. subtilis strain with the conditional knockout mutant genomic DNA, followed by positive selection for spectinomycin and negative selection for chloramphenicol. The full depletion of YdiB was checked using anti-YdiB antibodies obtained from Cocalico Biologicals. To monitor the growth of different strains, the strains were grown overnight on LB plates at 30 °C and one colony from each plate was used to inoculate an overnight liquid culture. On the following day, the cells were diluted into 200 μl LB medium in a 96-well microtiter plate at an initial OD600 of about 0.001. Samples were incubated at 30 °C with shaking at 250 r.p.m. for 26 h and the OD600 was measured at different times. For the conditional knockout strain, a growth analysis in the presence and absence of xylose was performed to assess the inducer dependence in liquid medium.

Construction of a B. subtilis K41A mutant strain. This strain was constructed by insertion of a ydiB variant (K41A) fused to a kanamycin-resistance cassette at the ydiB locus on the chromosome using the plasmid pBluescript (Stratagene) and the conditional knockout strain. ydiB flanking sequences were amplified by PCR from chromosomal DNA. Primers P1 (5′-GGAGGATCCATCTGTT-CTTCGCAACGGGCG-3′) and P2 (5′-GCTCGGCCCAGGGCGTT- AAACGTACCCGTCTTTTTAAAATG-3′) were used to amplify the downstream flanking sequence and primers P3 (5′-GAGCGGGAAATTACCTTTACG-3′) and P4 (5′-GAGGAAATCCCTAAAACACAGGGGGC-3′) were used to amplify the upstream flanking sequence. Primers kan-F (5′-CTGTTATTTAGGTATTAGAATGC-3′) and kan-R (5′-CTAAAACATTTATCGCAGAATTT-3′) were used to amplify a kanamycin-resistance cassette from pUK19. The K41A-ydiB gene was amplified from pET15b-K41A-ydiB (see below) using primers ydiB-F (5′-ATGGTGAAGCAATTAA-3′) and ydiB-R (5′-CTACTTGAATATTGTCACTT-3′). All the PCR products were amplified using Vent polymerase, purified and cloned into pBluescript vector. The upstream flanking sequence was cloned into the Smal site and then the downstream flanking sequence was cloned into the EcoRV site of the plasmid. As primer P2 contained Smal and Pmel sites, the kanamycin-resistance cassette could be cloned into the Smal site and the mutated gene into the Pmel site. The resulting plasmid contained 1 kb flanking sequences for the ydiB gene with the ydiB gene fused to a kanamycin-resistance cassette in between. This plasmid was then transformed into the conditional knockout strain, and a strain positive for chromosomal integration of K41A-ydiB and a kanamycin-resistance cassette at the ydiB locus was selected.

Construction of the overexpression plasmid pET15b-ydiB. ydiB was amplified by PCR using Pfu and B. subtilis DNA with the following primers: 5′-GGGCTCATCATCATAATGCTATATTGTCA-TGCTAC-3′ and 5′-GGGATCATATGGAAGCAGAATTTAATGAGA-GAAC-3′, with the underlined sequences corresponding to the restriction sites of BlpI and Ndel, respectively, and the stop codon being in bold. The gene was subsequently cloned into the BlpI and Ndel sites of pET15b (Novagen). The resulting plasmid (pET15b-ydiB) encodes the YdiB protein fused at its N-terminal end to a His, tag followed by a thrombin cleavage site. A variant of YdiB (K41A) was constructed with the Quick Change site-directed mutagenesis kit (Stratagene) using the pET15b-YdiB expression vector as a template. To screen for positive clones, oligonucleotides were designed to introduce the desired mutation and to simultaneously introduce a new Ndel restriction site (in bold) without modifying the protein sequence: 5′-GGGGATTATTTAGTGCGCCGGCCACGCTTTTACG- AAGG-3′ (the mutated bases are underlined). A second primer was used with a complementary sequence to this first primer. The correct
sequences of wild-type and mutant genes were verified by DNA sequencing (Genome Express).

**Production and purification of YdiB.** *E. coli* strain BL21(DE3) (Novagen) was transformed with the plasmid pET15b-**ydiB**. Cultures in 1 L LB medium with 100 μg ampicillin ml⁻¹ were grown at 37 °C until the OD₆₀₀ reached 0.6, induced by 1 mM IPTG, and further grown for 4 h. Cells were harvested by centrifugation, and resuspended in 20 ml lysis buffer (50 mM HEPES/KOH pH 7.5, 10 mM NaCl, 5 mM β-mercaptoethanol) supplemented with 1 mM PMSF, 5 μM leupeptin and 5 μM pepstatin A. The lysate was disrupted twice at 18 000 p.s.i. (124 MPa) in a French press apparatus, and cell debris was pelleted by centrifugation at 9000 g for 30 min at 4 °C. The clarified lysate containing YdiB was first purified by an anion-exchange step: 10 ml DEAE-cellulose slurry (Sigma), equilibrated in lysis buffer, was added to the clear lysate, and the solution was mixed gently at 4 °C for 60 min. The resin was then washed three times with a washing buffer (50 mM HEPES/KOH pH 7.5, 50 mM NaCl and 5 mM β-mercaptoethanol), and the beads were resuspended in an elution buffer (50 mM HEPES/KOH pH 7.5, 5 mM β-mercaptoethanol and 500 mM NaCl) and incubated for 30 min at 4 °C. After centrifugation, the supernatant was collected and loaded onto a nickel chelate chromatography column (Qiagen) at 4 °C. The column (Econo-pac from Bio-Rad) was allowed to drain (flow-through fraction), washed with 100 ml buffer (50 mM HEPES/KOH pH 7.5, 5 mM β-mercaptoethanol, 300 mM NaCl and 20 mM imidazole) and eluted with a buffer consisting of 50 mM HEPES/KOH pH 7.5, 5 mM β-mercaptoethanol, 100 mM NaCl and 250 mM imidazole. The purified protein was precipitated with ammonium sulphate at 55% saturation and stored at 4 °C. Using this procedure, 20–40 mg YdiB was routinely recovered. The purity of the recombinant protein was analysed by 14% polyacrylamide gel electrophoresis and the protein concentration was determined by the Bradford protein assay (Coomassie plus, Pierce), using BSA as a standard.

Before use, the precipitated protein in ammonium sulphate was centrifuged for 15 min at 15 000 g and 4 °C, and the pellet was resuspended in an appropriate buffer for the size-exclusion chromatography step (see below).

**Size-exclusion chromatography.** Size-exclusion chromatography experiments were performed at 4 °C on a Superdex 75 10/300 GL column (Amersham Biosciences), equilibrated with 50 mM HEPES/KOH pH 7.5, containing different concentrations of NaCl as indicated. The samples were centrifuged for 10 min at 18 000 g at 4 °C prior to loading 500 μl aliquots onto the column at a protein concentration between 1 and 5 mg ml⁻¹. Elution profiles were monitored by recording the A₂₈₀ using a flow rate of 0.5 ml min⁻¹, and different fractions were analysed by SDS-PAGE and used for enzyme assays. For calibration, BSA (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa) were used as molecular mass markers (gel filtration calibration kit LMW from Amersham GE Healthcare).

**Proteolytic cleavage using thrombin.** The pET15b plasmid allowed the fusion of a His₅ tag followed by a thrombin cleavage site at the N-terminus of the protein. When indicated, the His₅ tag was removed by incubation of 3 mg YdiB with 20 units thrombin at 4 °C for 2 h. Nickel-agarose resin was then incubated with the mixture for 30 min. The solution was centrifuged and the supernatant containing the YdiB protein without the tag or thrombin was used for gel-filtration experiments.

**SDS-PAGE and Western blot analyses.** For SDS-PAGE, the basic procedure was that of Laemmli (1970) under reducing conditions, using a 14% (w/v) acrylamide resolving gel, with a 4% stacking gel. Gels were stained with 0.2% Coomassie Brilliant Blue. Western-blot analyses were performed with either rabbit polyclonal anti-YjeE antibodies (dilution 1/10 000) or rabbit polyclonal anti-YdiB antibodies (dilution 1/10 000). To detect His-tagged proteins, the SuperSignal West Pico HisProbe kit (Pierce) was used, as described by the manufacturer.

**Native gel electrophoresis.** Nondenaturing PAGE was performed using a discontinuous buffer system. The resolving gel contained 370 mM Tris/HCl pH 8.8, 14% acrylamide, 0.1% TEMED and 0.1% ammonium persulphate. The stacking gel contained 70 mM Tris/HCl pH 6.8, 4% acrylamide, 0.1% TEMED and 0.1% ammonium persulphate. Gels were run in 50 mM Tris/HCl pH 8.4 and 400 mM glycerine at constant current (20 mA per gel) for 3–4 h at 4 °C, and then stained with 0.2% Coomassie Brilliant Blue.

**Ultracentrifugation analysis.** In order to remove ammonium sulphate and obtain an appropriate NaCl concentration, precipitated YdiB samples were centrifuged and the pellets were resuspended in a desired buffer and then equilibrated in the same buffer using a PD-10 column (from Amersham). Sedimentation velocity experiments were performed using a Beckman XL-I analytical ultracentrifuge equipped with an AnTi rotor. Samples and appropriate buffers (400 μl and 420 μl, respectively) were loaded into their respective channels in double-sector ultracentrifuge cells and run at 45 000 r.p.m. at 4 °C. Scans were recorded at 280 nm. The SEDFIT program continuous distribution c(s) analysis (Sedfit: http://www.analyticalultracentrifugation.com) (Dam & Schuck, 2004; Schuck, 2000) allowed us to fit the data by generating the sedimentation distribution profiles.

Mass identification of each peak was done by solving the sedimentation equation S(s)=<R²>/M=Mr(1−v_w/v_s), where s is the sedimentation coefficient of the analysed species, with calculated solvent viscosity ρ, density δ, estimated protein mass M and specific partial volume v_s. Considering YdiB as a globular protein, the Stokes radius R_s could be estimated by R_s=1.27 R_g (Damaschun et al., 1993). The gyration radius R_g was deduced from the known structure of YjeE (PDB: 1H9).

**ATPase assay.** An enzymic assay was used which couples the regeneration of ATP from the ADP produced to the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK) and the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). Hence, one mole of ATP hydrolysed is directly converted to one mole of ADP, PEP, and pyruvate, which is oxidized to NADH oxidized to NAD⁺, and the ATPase activity is monitored by the disappearance of NADH, followed at 340 nm. A typical reaction mixture (750 μl) contained 50 mM HEPES/KOH pH 7.5, 30 mM KCl, 4 mM PEP, 40 μg PK ml⁻¹, 20 μg LDH ml⁻¹ and 0.4 mM NADH (Jault et al., 1991) with various amounts of protein and the indicated salt and ATP plus MgCl₂ concentrations. The ATPase activity was monitored at 37 °C. K_m and V_max values were determined from iterative nonlinear fits of the theoretical Michaelis–Menten equation to the experimental data, using the GraFit 5.0.11 software (from Erithacus software).

**Formaldehyde cross-linking in vivo.** *E. coli* strain EB437 was used for this experiment. This strain contains an additional copy of the *araBAD* locus, which is under the control of the tightly regulated F_BAD promoter (Allali-Hassani et al., 2004). Bacteria were grown in LB liquid medium supplemented with 0.001% arabinose until the OD₆₀₀ reached ~1. Cross-linking was performed as previously described (Prossnitz et al., 1988; Skare et al., 1993), on bacteria resuspended in 100 mM sodium phosphate buffer pH 6.8 at OD₆₀₀ ~0.7. Formaldehyde (Fisher Chemical; 37%, w/w) was added at a final concentration of 1%, followed by incubation at room temperature. Samples (1 ml) were taken at the times indicated, and centrifuged immediately to pellet the whole cells. Pellets were washed...
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once in the same buffer, and then resuspended in concentrated (4 ×)
Laemmli sample buffer (250 mM Tris/HCl pH 6.8, 12 % SDS, 40 %
glycerol, 5 % β-mercaptoethanol and 0.01 mg bromophenol blue
ml⁻¹). The samples were heated either at 60 °C for 10 min to
maintain the formaldehyde cross-links or at 95 °C for 20 min to
break the chemical cross-links. Protein samples were analysed by
SDS-PAGE (14 % acrylamide), transferred to PVDF membrane
(Immobilon-P Transfer Membrane, Millipore) and then revealed by
immunoblotting using anti-YjeE antibodies.

Immunofluorescence microscopy. Cells harvested at an OD₆₀₀ of
~0.6 were fixed overnight at 4 °C in 4 % (v/v) paraformaldehyde,
30 mM sodium phosphate (pH 7.5) and washed three times in PBS.
Lysozyme was added to a final concentration of 2 mg ml⁻¹ for 2 min
and cells were transferred onto poly-l-lysine-coated Poly Prep slides
(Sigma). The slides were washed twice with PBS, air-dried, dipped in
methanol at −20 °C for 10 min and allowed to dry again. After
rehydration with PBS, the slides were blocked for 1 h at room
temperature with 5 % (w/v) milk in PBS (saturation buffer) and for
1 h with anti-YdiB antibodies diluted at 1:50 in saturation buffer.
The slides were then washed for 10 min in PBS and incubated with a
1:300 dilution of Cy3-conjugated goat anti-rabbit immunoglobulins
G (Jackson Immunoresearch) in saturation buffer. For DNA staining,
the slides were incubated with 2 µg DAPI ml⁻¹ (from TEBU) in
water. After extensive washing with PBS, the slides were mounted
using Mowiol solution. Samples were observed and photographed
with an Olympus fluorescence microscope equipped with a ×100
immersion objective.

RESULTS

A ydiB deletion strain grows slowly on a rich medium

Contradictory results have been reported regarding the
essentiality of YdiB in B. subtilis (Hunt et al., 2006;
Kobayashi et al., 2003), so we reinvestigated this point. A
conditional knock out strain was created using a method
previously described (Bhavsar et al., 2001b). Gene ydiB was
deleted from its original wild-type location on the
chromosome and a rescue copy was placed under the
control of a xylose-inducible promoter at the
amyE locus (amyE::ydiB Cm ydiB::spec). Fig. 1(a) shows a compar-
ison of the growth obtained in rich medium for the wild-
type B. subtilis and the conditional knockout mutant. After
a very short lag period (≤ 1 h), the wild-type grew steadily
with a doubling time of ~30 min to reach a plateau at
~8 h. For the conditional knockout mutant grown in the
absence of inducer, an increase in the lag phase (~3 h) and
a decrease in the growth rate (doubling time of ~110 min)
during the exponential phase were observed (the plateau
was reached at ~16 h). Addition of inducer (2 % xylose)
barely reduced the lag period but, once growth started, it
permitted a nearly normal growth rate of the mutant
(doubling time of ~35 min). These results suggested that
YdiB was dispensable in B. subtilis, although we could not
rule out that a slight leak of the promoter might account
for the slow growth of the mutant observed in the absence
of inducer. Therefore, we attempted to make a disrupted
ydiB mutant by transformation of the wild-type B. subtilis
strain with genomic DNA from the conditional mutant,
followed by spectinomycin selection. Our efforts resulted
in the creation of a disrupted ydiB mutant strain (inset
Fig. 1a), which grew very slowly in rich medium, at a rate
comparable to that of the conditional mutant in the
absence of xylose (Fig. 1a). This showed that the presence
of YdiB was not essential to the growth of B. subtilis in
a rich medium, but rather that the expression of YdiB was
required for rapid growth of the bacterium. Growth
experiments were also performed on minimal medium
(M9 supplemented with 50 µg tryptophan ml⁻¹) and
a similar trend was observed, although less pronounced,

Fig. 1. Growth of wild-type, ydiB-depleted cells and K41A ydiB
mutant in liquid or on solid media. (a) Wild-type (○) and disrupted
mutant (□) strains were grown overnight on LB plates and used to
inoculate LB medium. Growth was followed at 30 °C for 26 h. The
conditional knockout mutant was similarly grown in the presence
(■) or absence (▲) of 2 % xylose. The inset shows a Western blot
of B. subtilis lysates of wild-type (wt) and disrupted mutant (KO)
revealed by anti-YdiB antibody. A loading control using anti-TagD
antibody (Tag D is involved in the biosynthesis of cell wall teichoic
acid of B. subtilis; Bhavsar et al., 2001a) was also performed. (b)
Strains were grown overnight at 30 °C on an agar plate in the
presence (left panel) or absence (right panel) of 2 % (w/v) xylose.
Clockwise from top: wild-type (WT), disrupted mutant (KO), K41A
YdiB mutant (K41A), conditional knockout mutant (cKO).

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K41A mutant protein was overexpressed in B. subtilis grown in LB medium were analysed by Western blotting. Similar levels of YdiB were detected in each phase of growth, from the lag to the stationary phase (data not shown). To estimate the quantity of YdiB present in B. subtilis cells, a Western blot was performed and the intensity of the band revealed by the anti-YdiB antibody was compared to the intensity of the bands of increasing concentrations of purified YdiB (see below for the purification of YdiB). This led to an approximate concentration of YdiB of 0.4 μg per mg total protein (~0.04% of total protein).

In order to check if the putative ATPase activity of YdiB was required for normal growth of B. subtilis, the wild-type ydiB gene was replaced by a K41A ydiB variant, in a conditional knockout mutant background. The lysine mutated corresponds to the invariant residue found in the Walker A motif (G/AX4G, residue in bold) of many ATPases and GTPases (Geourjon et al., 2001; Saraste et al., 1990), and its replacement by other residues severely impaired the function of these enzymes (Reinstein et al., 1990; Shen et al., 1994). No growth was observed overnight on an agar plate in the absence of inducer for either the conditional knockout mutant or the mutant bearing the K41A mutation, showing that this mutation was not able to rescue the growth defect (Fig. 1b). This lack of rescue was not due to incorrect folding of the mutant protein because (i) when growth lasted long enough (after 2 days), the K41A mutant protein was detected by Western blotting in the soluble fraction of the strain, at a level similar to that obtained with the wild-type YdiB (not shown), and (ii) the K41A mutant protein was overexpressed in E. coli and purified with a similar yield as the wild-type YdiB, with the same ability to oligomerize (see below). Therefore, the lack of rapid growth of the strain carrying the K41A ydiB variant could be confidently attributed to a defect of ATPase activity of the mutant protein.

YdiB forms oligomers in vitro

The ydiB gene from B. subtilis was cloned into a pET15b vector, allowing the fusion of a poly-histidine tag at the N-terminus of the protein, and recombinant YdiB(His6) was overexpressed in E. coli and purified in soluble form. The expression level of wild-type YdiB(His6) was high, and the purification was carried out in two successive chromatography steps. A yield of 20–40 mg protein per litre of culture was routinely obtained and SDS-PAGE analysis revealed that the purified recombinant protein was nearly homogeneous (not shown). To further increase the purity of the protein, size-exclusion chromatography was employed. As shown in Fig. 2(a), YdiB was resolved as two major peaks during this step. Peak B (fractions 25–28) eluted at the volume expected for the monomeric form of YdiB (~20 kDa) given that the protein presumably has an overall globular shape (Teplyakov et al., 2002), whereas peak A (fractions 18–22) most likely corresponded to a mixture of dimers of YdiB and oligomers of higher molecular mass. That YdiB was indeed the major protein found in all these fractions, from peaks A and B, was confirmed by SDS-PAGE with Coomassie staining (Fig. 2b) and by Western blotting using an anti-His antibody (Fig. 2c). Interestingly, samples from peak A migrated in the SDS-PAGE as a mixture of YdiB monomers and SDS-resistant dimers. This surprising result, which was confirmed using the anti-His antibody, suggested that some YdiB dimers maintained their quaternary structure despite the harsh conditions of sample preparation and electrophoresis (exposure to 3% SDS in the loading buffer plus 160 mM β-mercaptoethanol and 0.1% SDS in the running buffer). When either peak A or B was rechromatographed by gel filtration, two major peaks were again observed, suggesting that, in solution, YdiB existed as an equilibrium between monomers and oligomers of higher molecular mass (data not shown). Further support for this conclusion came from native PAGE analysis of the fractions obtained from the gel filtration (Fig. 2d). Under these conditions, as expected, fractions from peak A (20–22) contained a mixture of oligomers of YdiB of different sizes that migrated as a ladder of species of increasing molecular masses. Oligomers were also observed when fractions from peak B (25–26) were analysed by native PAGE, which due to their elution times from the size exclusion chromatography were expected to contain only the monomeric form of YdiB. Again, this suggested an equilibrium among monomers and oligomers, and that the interconversion of the different molecular species of YdiB occurred on a relatively short timescale. A control was performed where the His6 tag was removed from YdiB by proteolytic cleavage using thrombin (as checked by SDS-PAGE due to a different size of the tagged and untagged YdiB) and this did not modify the behaviour of the protein, which still migrated as a mixture of monomers and dimers/oligomers on gel filtration (data not shown), showing that the presence of the His6 tag did not influence the equilibrium.

The oligomerization of YdiB is modulated by the salt concentration

Purified YdiB was analysed by size-exclusion chromatography over a large salt concentration range, from 20 to 300 mM NaCl (Fig. 3). In the presence of 20 mM NaCl, an elution profile similar to that seen in Fig. 2(a) was obtained, except that the first peak was clearly split into a doublet. The presence of this doublet, reflecting the occurrence of two main populations of oligomers of YdiB, was sometimes observed, depending on the batch of purified YdiB used. Increasing the concentration of salt progressively reduced the amount of material recovered in the doublet while it increased the quantity of YdiB...
recovered in the second peak. Simultaneously, this second peak was slightly shifted towards a longer elution time. This progressive shift in the elution time suggested that at low salt concentrations, there was a faster equilibrium between monomeric and dimeric forms of YdiB than at higher salt concentrations. On the other hand, in the presence of a high concentration of salt, the monomeric form of YdiB appeared much more stable and was therefore eluted from the column slightly later than at low salt concentrations.

To further characterize the oligomeric species formed by YdiB, an analysis was undertaken by analytical ultracentrifugation. Data from a typical sedimentation velocity experiment are shown in Fig. 4, where the analysis was performed in the presence of 500 mM NaCl. The experimental points (Fig. 4a) were fitted by a theoretical curve and the goodness of the fit was verified by both the random distribution of the residual values and the low values of the residuals (Fig. 4b). This indicated that a reliable model was obtained for the sedimentation velocity experiments. According to this model, most of the protein (~54%) sedimented with a value of 0.85 S (Fig. 4c), corresponding to one species with a mass of 20 kDa, in agreement with that predicted for the monomer. Two additional peaks were obtained at 1.45 S (~22%) and 2.3 S (~16%), corresponding to the predicted masses of the dimer (40 kDa) and tetramer (80 kDa), respectively, and a
very faint peak around 3.2 S (presence of some oligomers of higher molecular mass, ~8%). The lack of additional peaks at sedimentation coefficients up to 10 S indicated that the protein did not form aggregates. The proportion of each species at various NaCl concentrations was studied by sedimentation velocity experiments. As expected from the results previously obtained by size-exclusion chromatography, Fig. 5 shows that the ionic strength has a profound effect on the oligomerization properties of YdiB as monitored by analytical ultracentrifugation. At a low salt concentration (20 mM NaCl), the monomeric form of YdiB was a minority, amounting to less than 10 % of YdiB species, while there was a similar proportion of dimer and tetramer (~40 % each). When the salt concentration reached about 150 mM NaCl, a stable proportion of each species was observed, with 50–60 % of YdiB being monomeric, and ~25 % and ~15 % of YdiB being dimeric and tetrameric, respectively. This effect was not salt-specific since it was also observed using other salts such as KCl or Na₂SO₄ (data not shown).

The oligomerization of YdiB affects its ATPase activity

Previous reports have shown that orthologues of YdiB, namely YjeE from either E. coli or H. influenzae, exhibited a very low ATPase activity (Allali-Hassani et al., 2004; Teplyakov et al., 2002). Therefore, we investigated whether YdiB from B. subtilis also displays an ATPase activity and, given its propensity to oligomerize, we evaluated how this process might affect YdiB ATPase activity. For this purpose, a high salt concentration was used during the size-exclusion chromatography, for optimal resolution of the fractions of monomers from dimers/oligomers, and these two fractions were immediately assayed for their ATPase activity using an enzyme-coupled assay system. In this assay, the regeneration of hydrolysed ATP allows maintenance of a constant level of substrate while

![Figure 3. Effect of increasing concentrations of NaCl on the monomer/oligomer equilibrium.](image)

![Figure 4. Sedimentation velocity experiments and sedimentation distribution profile of YdiB.](image)
preventing product inhibition by the ADP produced, and thus an initial rate of ATPase activity is sustained over several minutes. Using a saturating concentration of ATP (5 mM, see below), the rate of ATP hydrolysis was about three times higher for the monomeric fractions as compared to the dimers/oligomers fractions [11 and 3.5 nmol min$^{-1}$ (mg protein)$^{-1}$, respectively]. It is important to note here that this magnitude very probably underestimates the true difference in ATPase activity between the two fractions, because on the timescale of this experiment, we could not prevent some interconversion of the dimers/oligomers into monomers after the size-exclusion chromatography step. Indeed, when the ATPase activity of the dimers/oligomers was monitored at different times after the gel filtration, a progressive increase of the activity was observed (data not shown). So far, we have been unable to stabilize the oligomeric state(s) of YdiB despite investigating a wide variety of experimental conditions (various temperatures and/or different salts or buffers), precluding a thorough characterization of the ATPase activity of this fraction. On the other hand, the ATPase activity of the monomeric fraction was rather stable over time. Nevertheless, our ultracentrifugation experiments (Fig. 5) indicated that even at a high salt concentration (500 mM NaCl), the monomer represented no more than 60% of the whole population of YdiB. Therefore, it is likely that after the recovery of the monomeric fraction of YdiB from the gel filtration column, a relatively fast equilibrium was reached where ‘true’ monomer amounted to ~60% of the whole population of YdiB.

With the oligomeric fraction being unstable over time, we focused our attention on the ATPase activity of the monomeric fraction. First, to rule out the possibility that this low activity was due to contaminant(s), several controls were performed. Addition of 1 mM GTP instead of ATP gave a very slow rate of hydrolysis (Fig. 6a, dashed line), similar to that obtained when no nucleotide was added (not shown), but much lower than that measured in

![Fig. 5. Effect of ionic strength on the quaternary structure of YdiB.](http://mic.sgmjournals.org)

The sedimentation of YdiB (1.2 mg ml$^{-1}$) was analysed by analytical ultracentrifugation as in Fig. 4, using various concentrations of NaCl as indicated, and the proportions of each species were estimated by integration of their corresponding peak. The species are as follows: monomers (●), dimers (□), tetramers (■) and oligomers of higher molecular mass (○).

![Fig. 6. Characterization of the ATPase activity of the monomeric fraction of YdiB.](http://mic.sgmjournals.org)

(a) ATPase activity of 130 μg of wild-type YdiB (plain line) or K41A variant (dotted line) was measured in the presence of 100 μM ATP and 100 μM MgCl$_2$, following the disappearance of NADH at 340 nm using an enzyme-coupled assay system (see Methods). The GTPase activity (1 mM GTP-Mg instead of ATP-Mg) was also monitored for the wild-type YdiB (dashed line). (b) ATPase activity as a function of the ATP concentration; 145 μg (10 μM) of YdiB from the monomeric fraction was used. The data were fitted to the Michaelis–Menten equation using the GraFit software, allowing the determination of the kinetic parameters: $K_m = 62.1 \pm 4.6$ μM and $V_{max} = 10.5 \pm 0.17$ nmol ATP hydrolysed min$^{-1}$ (mg protein)$^{-1}$ ($k_{cat} \sim 10$ h$^{-1}$). The inset shows the Lineweaver–Burk plot of the data with the fitted curve. One set of data is shown; similar results were obtained from three independent experiments.
the presence of ATP (Fig. 6a, solid line). This indicated that the ATPase activity measured in the presence of YdiB was neither due to some contaminant(s) with NADH dehydrogenase activity nor caused by some GTPase enzyme having a residual ATPase activity. In addition, a YdiB variant was created where the conserved Walker A lysine residue was mutated to an alanine (K41A mutant, see above), and the monomeric fraction of the variant was purified as for the wild-type, with a similar yield, degree of purity and oligomerization state (data not shown). This K41A variant exhibited an extremely low ATPase activity (Fig. 6a, dotted line) as compared to the wild-type enzyme, close to the background level, showing that this substitution led to an inactive enzyme. Therefore, the ATPase activity measured with the wild-type enzyme can be confidently attributed to an intrinsic property of YdiB.

The ATPase activity was then analysed as a function of the concentration of YdiB. A linear dependence was observed between the rate of ATP hydrolysis and the concentration of YdiB. A linear dependence was observed (Fig. 6b). The values of the kinetic parameters, $K_m$ and $V_{max}$, were determined from the fitted curve as being $62.1 \pm 4.6 \mu M$ and $10.5 \pm 0.17$ nmol ATP hydrolysed min$^{-1}$ (mg protein)$^{-1}$ ($k_{cat} \sim 10$ h$^{-1}$), respectively.

YjeE, the YdiB orthologue from *E. coli*, also forms oligomers *in vitro*

In order to investigate whether the oligomerization of YdiB was a unique property of the *B. subtilis* enzyme, or if it was a common property shared with other members of the same family, YjeE from *E. coli* was purified as previously described (Allali-Hassani *et al.*, 2004). When 5 µg of purified YjeE was subjected to native PAGE, the protein was resolved as a ladder of at least four bands corresponding to different states of oligomerization (Fig. 7). It should be noted that in this kind of electrophoresis, one cannot directly compare the profile of migration of YdiB (Fig. 2d) and YjeE (Fig. 7) because the migration is not strictly proportional to the size of the protein (or its oligomers) as it depends also on the charge of the protein (Speed *et al.*, 1995).

**YdiB or YjeE probably forms oligomers *in vivo***

We then asked whether this class of proteins can form oligomers *in vivo*. To investigate this, a formaldehyde cross-linking approach was first used. This small reactive molecule, capable of polymerization, can be used in whole cells to reflect protein–protein interactions as they occur *in vivo* (Peters & Richards, 1977). Furthermore, the cross-linked products formed can be destroyed by subsequently heating the samples. Fig. 8(a) shows the results obtained when *E. coli* cells expressing YjeE, treated with 1% formaldehyde for various times, were analysed by SDS-PAGE and the protein immunodetected using anti-YjeE antibodies. YjeE was present in all the samples, as expected, and migrated with an apparent molecular mass of ~17 kDa (Fig. 8a, lanes 1 to 4) and this band was the only one revealed in the control experiment (lane 1). An additional band with an apparent molecular mass of ~35 kDa, corresponding approximately to the expected size of a dimer of YjeE, was detected immediately after formaldehyde addition and its intensity increased after 1 h of incubation (lanes 2 and 3, respectively). This band essentially disappeared after boiling (lane 4), showing that it was indeed due to a cross-link promoted by formaldehyde. A minor band corresponding putatively to a tetramer (apparent molecular mass of ~60 kDa) was also detected immediately after formaldehyde addition (lane 2) but this band seemed to vanish in the sample incubated for 1 h (lane 3). Surprisingly, however, in this 1 h incubation, a band with a quite high molecular mass, not capable of penetrating through the gel, was detected. This species too disappeared after the subsequent boiling step (compare lanes 3 and 4). This might possibly correspond to a high molecular mass assembly of YjeE which was revealed only after 1 h of incubation in the presence of formaldehyde. If it were the case, it is possible that immediately after formaldehyde addition, some tetramers belonging to larger oligomeric assemblies were immediately cross-linked (lane 2) but after the 1 h incubation period, additional cross-links led to the disappearance of the tetramer and, concomitantly, to the occurrence of cross-linked species.
of higher molecular mass. The smear observed in lanes 2 and 3 also warrants some comments here. These species presumably corresponded to oligomers (dimers or higher molecular mass species) cross-linked more than once with different formaldehyde molecules. Alternatively, some of these bands might correspond to YdiB cross-linked to as yet unknown physiological partners, proteins or nucleic acids. In order to substantiate the formation of oligomers in vivo, total extracts of B. subtilis were analysed by Western blotting using anti-YdiB antibodies (Fig. 8b). While no YdiB was observed in the knockout mutant as expected (lane 2), the monomer was detected in the wild-type strain. More importantly, a band presumably corresponding to the dimers was also detected in wild-type B. subtilis (lane 1), and this very likely confirms that, in vivo, the protein retains its capacity to form oligomers.

**Cellular localization of YdiB**

Lastly, immunofluorescence experiments were performed on both wild-type B. subtilis and the mutant bearing the K41A mutation to identify the cellular localization of YdiB. Fig. 9 shows that YdiB appears to be unevenly distributed in the wild-type cells, being predominantly located at the periphery of the bacterium and at the poles; it therefore seems to be excluded from the nucleoids. A similar pattern of distribution was observed with the K41A mutant of YdiB, which sometimes appeared somewhat more intense at the poles of the cells.

**DISCUSSION**

Gene dispensability has been a recurrent question in the post-genomic era of microbiology. The ydiB gene was initially defined as being essential in B. subtilis based on failed attempts at gene disruption (Kobayashi et al., 2003). More rigorous studies in E. coli led to an essential designation for the orthologous gene yjeE (Allali-Hassani et al., 2004; Freiberg et al., 2001). Most recently, however, a viable knockout mutant of B. subtilis ydiB was reported, suggesting that ydiB was not ‘essential’ sensu stricto (Hunt et al., 2006). Our results agree with this latter study but clearly show that a ydiB knockout mutant is profoundly impaired for growth compared to the wild-type strain. Thus, the notion of ‘essentiality’ for a gene perhaps deserves another boundary rather than just its ability to allow some growth, or not, on a defined medium; this point has been thoroughly discussed recently by Danchin and colleagues (Fang et al., 2005). Accordingly, the conservation of a gene among the majority of bacteria, even if proven nonessential for growth on rich laboratory media, is grounds to classify a gene as critical to cellular viability. Genes of this type, called ‘persistent nonessential’, might play a crucial role when bacteria have to cope with hostile conditions, especially in the wild, where they thrive under nutrient-limited conditions while they compete with other microbes. Considering this definition, it is clear that
ydiB or its orthologues, which are conserved in almost all bacteria, have critical though currently elusive functions in bacterial physiology.

A key discovery reported here is the propensity of YdiB and YjeE to form higher oligomers in vitro, and possibly in vivo. In support of this conclusion, it is noteworthy that using a tandem affinity purification tag approach, Butland et al. (2005) found that E. coli YjeE interacts with itself. On the other hand, crystal structures of YjeE from H. influenzae, either the apo- or the ADP-Mg bound enzyme, were shown to be monomeric in the crystal (Teplyakov et al., 2002). It is worth noting that both structures were, however, obtained in the presence of a high concentration of salts, shown here to discourage the oligomerization of the YdiB protein.

The salt concentrations that affected the oligomerization status of YdiB in vitro appear to be in the range of the physiological concentration of salts found in bacteria (Teixeira de Mattos & Neijssel, 1997), e.g. at least 150–200 mM K⁺ in E. coli and ~400 mM K⁺ in B. subtilis, consistent with a possible modulating effect on the ATPase activity of YdiB in vivo. Because high ionic strength has a profoundly negative effect on the oligomerization of the protein, this suggests that, at the molecular level, interactions between different monomers mainly involve charged, or polar, residues. This feature might explain why the oligomers, notably the dimers, of YdiB were partially resistant to SDS. Indeed, if the interfaces between monomers were stabilized by a network of polar interactions, including negatively charged residues, this would possibly result in an unfavourable interaction with SDS, thereby leading to SDS-resistant oligomers. Such a scenario has been proposed before for the β-glycosidase from the hyperthermophilic bacterium Sulfolobus solfataricus (Gentile et al., 2002).

The ability to dimerize or oligomerize appears to be a property shared by many proteins (Park et al., 2001). In the case of enzymes, this feature is generally associated with the ability to tightly control the catalytic activity, and this may explain the apparent negative effect of the oligomerization reported here on the ATPase activity of YdiB. Many P-loop ATPases have been shown previously to function as oligomers inside the cell, including for instance the AAA proteins (for ‘ATPase Associated with many cellular Activities’, Lupa & Martin, 2002), the ParA superfamily involved in bacterial DNA segregation (Barilla et al., 2005) or MinD, which spatially regulates cell division in E. coli (Hu et al., 2003). However, and in contrast to what was found here for YdiB, the oligomerization of these P-loop ATPases always increases their specific activity. Usually, when these P-loop enzymes couple ATP hydrolysis to a given cellular function, their ATPase activity remains low in the absence of the cellular partner (e.g. membrane-bound components for members of the secretion NTPase superfamily; Crowther et al., 2005) or the physiological substrate (e.g. DNA for helicase; Wong et al., 1996). As shown here, YdiB has a low ATPase activity (~10 h⁻¹) consistent with the low activity reported previously for YjeE from either E. coli (12 h⁻¹; Allali-Hassani et al., 2004) or H. influenzae (1.2 h⁻¹ or 25 h⁻¹ according to the authors; Allali-Hassani et al., 2004; Teplyakov et al., 2002). Importantly, this low ATPase activity is shown here to be essential for the in vivo function of the YdiB protein because, due to its lack of ATPase activity, the K41A YdiB mutant is unable to restore a normal growth phenotype. Until now, the cellular function of YdiB/YjeE has remained

![Fig. 9. Cellular localization of YdiB. B. subtilis wild-type (a, d, g) or mutant strains, K41A (b, e, h) or knockout (c, f, i), were grown in LB medium. Nucleoids were stained with DAPI (blue colour in a, b and c) and YdiB proteins were detected by immunofluorescence (red colour in d, e and f); overlays of the two images are shown in g, h and i. Images taken at ×100 and reproduced at ×70.](image-url)
elusive but it is likely that the low ATPase activity of this new class of protein will be stimulated by the presence of its physiological partner, once identified. In this regard, it will be of major importance to check how the oligomerization status of YdiB will affect the interaction with its partner. Based on the phylogenetic distribution of the YdiB/YjeE family in all bacteria except Mycoplasma and Ureaplasma, it has been suggested that it plays a role in cell-wall biosynthesis (Teplyakov et al., 2002). In support of this hypothesis, yjeE is present in some species in the same operon as amiB, which encodes an amidase involved in recycling of peptidoglycan, and both genes are transcribed together in E. coli (Tsui et al., 1994). Using different co-purification approaches to identify protein–protein partners on a wide genomic scale, two recent studies reported several but different putative protein partners for YjeE in E. coli, thus making it difficult to rationalize the possible function of this protein inside the cell (Arifuzzaman et al., 2006; Butland et al., 2005). The cellular localization reported here might help to give some hints about the role of the YdiB/YjeE protein family. Using a GFP-tagged YdiB under the control of the xylose promoter, it was previously reported that the protein was uniformly dispersed over the entire cell and therefore has a cytoplasmic localization (Hunt et al., 2006). Here, by immunofluorescence and using the native protein, the localization appears restricted to the cytoplasmic area where the DNA is missing, with a possible preference for the cell poles. This cellular localization has been reported previously for some proteins involved, for instance, in septation, such as MinD in B. subtilis (Marston et al., 1998). Also, ribosomes have been shown to be preferentially located at the cell poles (Lewis et al., 2000). Interestingly, in the two reports mentioned above using a co-purification approach to identify protein partners, several ribosomal proteins were proposed as putative partners of YjeE in E. coli (Arifuzzaman et al., 2006; Butland et al., 2005). Clearly, further experimental evidence will be required in order to decipher the precise nature of the cellular role of the bacterial YdiB/YjeE family but the ATPase activity, modulated by the oligomeric status of the protein, will certainly be an essential feature for its function.

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